FEBS 29434 FEBS Letters 579 (2005) 2191–2196

Identification of the reactive cysteine residues in oligopeptidase B from *Trypanosoma brucei*

Rory E. Morty[a,*], Angela Y. Shih[b], Vilmos Fülöp[c], Norma W. Andrews[b]

[a] *Department of Internal Medicine, University of Giessen School of Medicine, Aulweg 123 (Room 6-11), D-35392 Giessen, Germany*
[b] *Section of Microbial Pathogenesis, Boyer Center for Molecular Medicine, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06536, USA*
[c] *Department of Biological Sciences, University of Warwick, Gibbit Hill Road, Coventry CV4 7AL, United Kingdom*

Received 17 January 2005; revised 7 March 2005; accepted 7 March 2005

Available online 17 March 2005

Edited by Stuart Ferguson

Abstract Oligopeptidase B (OpdB) from *Trypanosoma brucei* is a candidate therapeutic target in African trypanosomiasis. OpdB is an atypical serine peptidase, since activity is inhibited by thiol-blocking reagents and enhanced by reducing agents. We have identified C256 as the reactive cysteine residue that mediates OpdB inhibition by *N*-ethylmaleimide and iodoacetic acid. Modeling studies suggest that C256 adducts occlude the P_1 substrate-binding site, preventing substrate binding. We further demonstrate that C559 and C597 are responsible for the thiol-enhancement of OpdB activity. These studies may facilitate the development of specific OpdB inhibitors with therapeutic potential, by exploiting these unique properties of this enzyme.

Keywords: Trypanosomiasis; Oligopeptidase; Peptidase; Chemotherapy; Reductive activation

1. Introduction

African trypanosomes contain a serine oligopeptidase, oligopeptidase B (OpdB) that cleaves oligopeptides on the C-terminal side of basic residues [1], and is implicated in the pathogenesis of African trypanosomiasis, since it is released into the bloodstream of infected mammalian hosts [2]. Since OpdB is not inhibited by endogenous serine peptidase inhibitors in the host bloodstream [3], it retains catalytic activity and thus participates in the pathogenesis of African trypanosomiasis through anomalous degradation of host peptide hormones [4].

Oligopeptidase B from *T. b. brucei* has also been identified as a target of several drugs used to treat African trypanosomiasis. It is inhibited by the aromatic diamidines pentamidine and diminazene, and the sulfonated naphthylurea, suramin [5]. Irreversible inhibitors of OpdB, including several peptidyl chloromethylketones and peptidyl α-aminoalkyl phosphonate diphenyl esters exhibited anti-trypanosomal activity, both in vitro and in vivo [6]. These findings suggested that the potential of OpdB as a therapeutic target warranted further study. However, the P_4–P_1 specificity of OpdB parallels that of many mammalian plasma serine peptidases, including members of the blood clotting and complement cascades [1,7]. This parallel specificity of OpdB and several trypsin-like serine peptidases in the host plasma has hampered our efforts to develop specific OpdB inhibitors with therapeutic potential. For example, the peptidyl α-aminoalkyl phosphonate diphenyl esters referred to above also prolong the prothrombin and activated partial thromboplastin time in mice [8], probably accounting for the observed toxicity of these compounds when used to treat experimental trypanosome infections in a rodent model [6]. These findings have led us to explore other unique physico-chemical properties of OpdB, which distinguish OpdB from other mammalian plasma serine peptidases, in the hope that these properties will facilitate the development of specific inhibitors that selectively target OpdB without affecting the activity of other mammalian host serine peptidases. One such property is the pronounced sensitivity of OpdB to reagents that react with the thiol groups of cysteine residues.

Unlike most serine peptidases, the activity of OpdB isolated from two important pathogenic African trypanosomes, *T. b. brucei* [1] and *T. congolense* [9] is inhibited by thiol-blocking reagents, and is enhanced by reducing agents applied at low millimolar concentrations. The mechanisms underlying these two phenomena have not been elucidated. The *T. brucei* primary sequence contains 14 cysteine residues [1] and it is likely that thiol-blocking reagents and reducing agents act on one or more of these 14 residues, thereby causing the observed inhibition and activation, respectively. In this study we set out to identify which cysteine residues of OpdB were responsible for mediating these effects.

2. Materials and methods

2.1. Isolation of OpdB from T. brucei

Oligopeptidase B was isolated from *T. b. brucei* ILTat1.1 lysates as described previously [1]. Active enzyme concentration was determined with 4-methylumbelliferyl-*p*-guanidinobenzoate [1].

*Corresponding author. Fax: +49 (641) 994 2309.
E-mail address: rory.morty@innere.med.uni-giessen.de (R.E. Morty).

Abbreviations: AMC, 7-amino-4-methylcoumarin; Cbz, carbobenzyloxy; DTT, dithiothreitol; GSH, reduced glutathione; IAA, iodoacetic acid; IAN, iodoacetamide; k_{ass}, apparent second-order inhibition rate constant; NEM, *N*-ethylmaleimide; OpdB, oligopeptidase B; pCMB, *para*-chloromercuribenzoate; POP, prolyl oligopeptidase; WT, wild-type

0014-5793/$30.00
doi:10.1016/j.febslet.2005.03.014

Table 1
Mutagenic primers used to introduce cysteine mutations into *T. brucei* OpdB

Mutant	Sequence
C117S	5′-GTACAAGCTTCATT**C**TCGCGTACCGGCC-3′
C147S	5′-GGCAAATCTTTTT**C**TGTCGTGGGTTGC-3′
C151S	5′-TGTGTCGTGGGT**C**CGTGGCGCCCGCC-3′
C169S	5′-TCCGTTGATTAC**A**GCGGGGATGAGGTG-3′
C202S	5′-CCCAATGCCGAA**C**CTTCTTTTACATTAC-3′
C231S	5′-GTGAAGATGTA**C**CCTCTACACCGAC-3′
C256A	5′-GCTGATCATT**GC**CTCTATGTCATC-3′
C256G	5′-GCTGATCATT**G**GCTCTATGTCATC-3′
C256S	5′-GACGCTGATCATT**C**CTCTATGTCATCAG-3′
C310S	5′-CAAATAAAGACAAG**C**CGTGAATGGTAAG-3′
C393S	5′-GCCCATGTGGTT**A**GTTCTCAAATGAAG-3′
C446S	5′-GAAGAATTACGTT**A**GTCGGAGGGAGTTG-3′
C488S	5′-CCTACGGCATTT**C**CATTGAGCCTGAG-3′
C542S	5′-GGACTTTATTGCAT**C**CGCGGAGCACC-3′
C559S	5′-GCGCAGCTTTCTT**C**CGAGGGAAGAAGTGC-3′
C597S	5′-CGTAATGACAACCATGT**C**TGATCCAAGCATTCC-3′

The Cys → Ser codon is underlined, and the base-pair changes are illustrated in bold type. Only the forward (*sense*) primer is indicated.

2.2. Site-directed mutagenesis

A pET19b::*opdB* expression plasmid for production of recombinant N-terminal polyhistidine-tagged *T. brucei* OpdB [1] was used for the generation of site-directed OpdB variants, performed as described previously [10], employing the mutagenic oligonucleotide primers in Table 1. Point mutations were confirmed by DNA sequencing from the pET19B T7 terminator with a universal T7 terminator primer (5′-GCT AGT TAT TGC TCA GCG G-3′), or using forward sequencing primers 5′-CTA CAC GCG TGA TGT AAA GGG-3′ or 5-CGG TGG GTG TGG GGA GGT CGG-3′, corresponding to base-pairs 219–240 and 632–652, respectively of the *T. brucei opdB* open reading frame [1]. In selected cases (the C256A, C256G and C256S constructs) the entire coding sequence was sequenced in both directions to check for undesired point mutations.

2.3. Hyper-expression of site-mutated OpdB variants

For expression, *opdB* mutant constructs were transformed into *Escherichia coli* BL21 λDE3 and recombinant protein expressed as described previously [10]. Protein purity was evaluated by Tris–Tricine SDS–PAGE. The OpdB variants (2 μg) were resolved on a 10% Tris–Tricine SDS–PAGE gel with or without prior reduction (boiling in 100 mM dithiothreitol (DTT)).

2.4. Determination of amidolytic activity

Activity of OpdB was determined against 5 μM *N*-carbobenzyloxy (Cbz)-L-Arg-L-Arg-7-amido-4-methylcoumarin (AMC) at 37 °C in 50 mM Tris–Cl/5 mM DTT, pH 8.0 in an Hitachi F-2000 spectrofluorimeter (λ_{ex} = 370 nm, λ_{em} = 460 nm) [1]. Kinetic analyses (k_{ass}, K_m and k_{cat}) were undertaken as described in [1]. Intergroup differences were estimated by a statistical analysis of variance (ANOVA). Fisher's protected least-significant difference test was employed to compare individual groups. Differences within the same group were evaluated with Student's *t* test.

2.5. Protein sequence alignments and molecular modeling

Protein sequences were aligned with the CLUSTAL W software from the MEGALIGN program (DNASTAR, Madison, WI), with a PAM250 weight table set with the following parameters: ktuple = 1, gap penalty = 3, and gap window = 5 [11]. Molecular modeling procedures are described in detail in [12], and are indicated in the legend to Fig. 3A.

3. Results

All recombinant OpdB variants migrated as a single band of ≈80 kDa on SDS–PAGE under reducing and under non-reducing conditions (data not shown). The K_m and k_{cat} values for native and recombinant wild-type (WT) OpdB were in excellent agreement, and both enzymes were inactivated by *para*-chloromercuribenzoic acid (pCMB), *N*-ethylmaleimide (NEM), iodoacetic acid (IAA) and iodoacetamide (IAN) to comparable extents (Fig. 1A). Thus, the poly-histidine affinity tag did not alter the enzymatic properties of OpdB.

All cysteine residues in the OpdB sequence were separately converted to serine residues. All site-mutated Cys → Ser variants were catalytically active against Cbz-Arg-Arg-AMC, exhibiting an average k_{cat}/K_m of 62 ± 14 s^{-1} μM (data not shown), when assayed in 50 mM Tris–Cl, pH 8, at 37 °C. Thus, the cysteine replacements did not appreciably impair the catalytic competence of the OpdB variants. The apparent second-order inhibition rate constant (k_{ass}) values for the association of all OpdB variants with IAA and NEM were comparable to the WT enzyme (k_{ass} values in the range of 1.5–2.5 M^{-1} s^{-1}), except for the C256S variant, which exhibited a 750-fold reduction in k_{ass} for IAA (0.0021 ± 0.0008 M^{-1} s^{-1}) and more than 1000-fold reduction in k_{ass} for NEM (0.0012 ± 0.0010 M^{-1} s^{-1}). Overall, pCMB exhibited a faster rate of association with OpdB variants than did IAA or NEM (average k_{ass} 20–30 M^{-1} s^{-1}), and again the C256S was more resistant to pCMB inhibition (16 ± 2 M^{-1} s^{-1}), however, this effect was less significant ($P < 0.05$) than were the

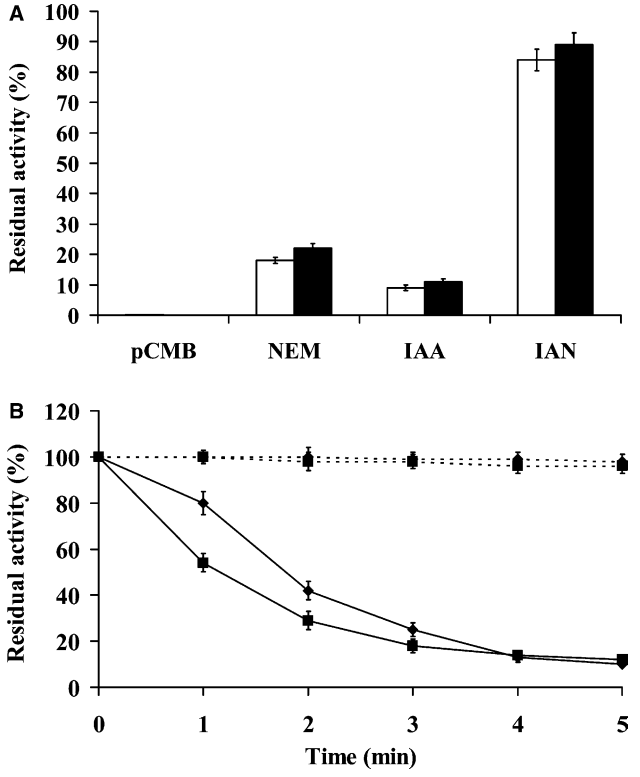

Fig. 1. Inhibition of OpdB by thiol-blocking reagents. (A) Native OpdB purified from *T. brucei* lysates (open bars) and recombinant His_6-tagged WT OpdB (closed bars) were pre-incubated with pCMB, NEM, IAA and IAN (10 mM), prior to assessment of hydrolytic activity. Data reflect mean residual activity ± S.D (n = 3). (B) The time-course for the inactivation of WT His_6-tagged OpdB (solid line) and its C256S variant (dashed line) by 5 mM NEM (■) or 5 mM IAA (◆). Data reflect mean residual activity ± S.D. (n = 3). WT, wild-type.

effects observed for IAA ($P < 0.001$) and NEM ($P < 0.001$). The C256S OpdB variant was remarkably resistant to inactivation by both NEM and IAA, demonstrating a negligible (1–2%) reduction in activity after a five min pre-incubation with either NEM or IAA at 5 mM (Fig. 1B). These data suggested that C256 was the reactive cysteine residue responsible for the inactivation of OpdB by NEM and IAA.

Serine (CH_2OH side-chain) is structurally very similar to cysteine (CH_2SH side-chain). Furthermore, serine and cysteine are both uncharged, polar residues, and are thus likely to have similar physico-chemical properties. Hence, we considered serine a good cysteine mimic, and routinely employed serine in our cysteine conversions. However, in the event that a serine substitution caused a relevant conformational change in OpdB, we also replaced C256 with an alanine residue (C256A) and a glycine residue (C256G). Both of these variants behaved in a manner comparable with the C256S variant, and exhibited k_{ass} values with IAA of 0.0037 ± 0.0011 and $0.0045 \pm 0.0024\ M^{-1}\ s^{-1}$ for the C256A and C256G variants, respectively. Similarly, in the presence of NEM, the C256A and C256G variants exhibited k_{ass} values of 0.0007 ± 0.0002 and $0.0005 \pm 0.0004\ M^{-1}\ s^{-1}$, respectively. These data support our contention that C256 is the key residue involved in mediating OpdB inhibition by IAA and NEM.

The activity of native and recombinant WT OpdB was enhanced by 5 mM DTT, glutathione (GSH) and L-cysteine (Fig. 2A), indicating that the poly-histidine affinity tag did not alter thiol-activation properties. Half-maximal enhancement of recombinant WT OpdB occurred at sub-millimolar DTT and GSH concentrations (≈0.63 mM; Fig. 2B). Under non-reducing and reducing (10 mM DTT) conditions, OpdB eluted from a Sephacryl S-200 HR molecular exclusion column at a molecular mass corresponding to 80 kDa, the size of monomeric OpdB (data not shown). Thus, OpdB does not form inactive multimers under non-reducing conditions.

The k_{cat}/K_m values for all OpdB variants treated with DTT and GSH were comparable to the WT enzyme, except for the C559S and C597S variants, which exhibited a significant ($P < 0.01$) reduction in the degree of activation. WT OpdB exhibited a k_{cat}/K_m value of $468 \pm 19\ s^{-1}\ \mu M$ in the presence of 5 mM DTT, while the k_{cat}/K_m values for the C256S, C559S and C597S variants were reduced to 418 ± 22, 190 ± 12 and $254 \pm 8\ s^{-1}\ \mu M$, respectively. Similarly, in the presence of 5 mM GSH, WT OpdB exhibited a k_{cat}/K_m value of $388 \pm 18\ s^{-1}\ \mu M$, while C256S, C559S and C597S variants were reduced to 312 ± 6, 194 ± 16 and $211 \pm 10\ s^{-1}\ \mu M$, respectively. A C559:597S double-variant exhibited a meagre two-fold elevation in k_{cat}/K_m in the presence of DTT, and 1.8-fold in the presence of GSH (Fig. 2C). Even less effect was seen in the C256:559:597S triple-variant, which exhibited a 0.8- and 0.7-fold elevation in k_{cat}/K_m in the presence of DTT and GSH, respectively (Fig. 2C). However, even this triple C256:559:597S OpdB variant still exhibited a significant ($P < 0.05$) increase in activity in the presence of 5 mM DTT and 5 mM GSH when compared with OpdB that was not treated with any reducing agent (Fig. 2C). These data confirm that C559, C597 and perhaps C256 are primarily responsible for the thiol-activated activity of OpdB, however, it appears that one or more additional cysteine residues contribute, albeit weakly, to this phenomenon.

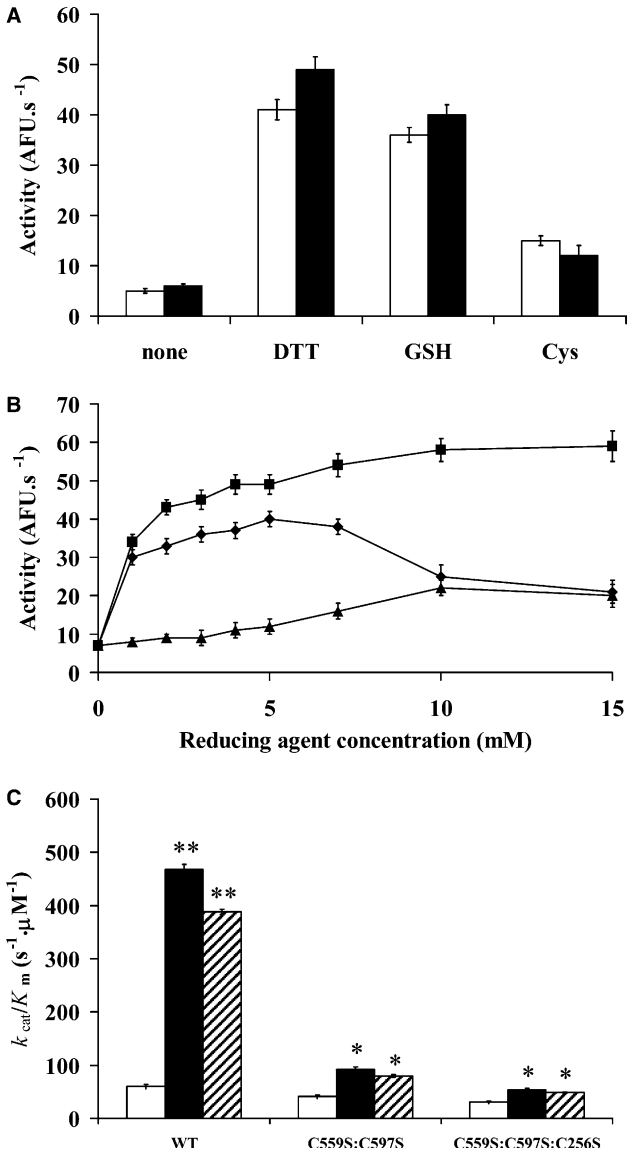

Fig. 2. Enhancement of OpdB activity by reducing agents. (A) Native OpdB purified from *T. brucei* lysates (open bars) and recombinant polyhistidine-tagged WT OpdB (closed bars) were pre-incubated with DTT, GSH or L-cysteine (Cys), after which residual activity against Cbz-Arg-Arg-AMC was determined. (B) Dose-dependence for the enhancement of OpdB activity by DTT (■), GSH (◆) and Cys (▲). In both panels, data reflect the mean activity ± S.D. ($n = 3$). (C) Recombinant polyhistidine-tagged OpdB variants (≈150–200 fmol active enzyme), were pre-incubated in the absence (open bars), or presence of 5 mM DTT (closed bars) or GSH (hatched bars) prior to assessment of hydrolytic activity. Data reflect the mean k_{cat}/K_m ± S.D. ($n = 5$). WT, wild-type. **, $P < 0.001$; *, $P < 0.05$, with respect to OpdB that was not treated with any reducing agent (open bars).

4. Discussion

Oligopeptidase B from *T. brucei* was potently inactivated by IAA and NEM, while IAN had less effect. We have previously speculated [1] that thiol-blocking agents may inhibit OpdB by alkylation of C559, which lies four residues N-terminal to the active-site serine residue (Fig. 1), and proposed that alkylation of C559 would impair catalysis given its proximity to the active-site serine. In the present study, we demonstrate that

inhibition by IAA and NEM was mediated exclusively by C256. Our original suggestion of C559 was therefore incorrect. The agents IAA and NEM do not inhibit OpdB from *T. cruzi* [13], *E. coli* [14] and *Moraxella lacunata* [15]. Interestingly, OpdB homologues from these three organisms do not have a cysteine residue at position C256, where it is replaced by a glycine, tyrosine and histidine residue, respectively (Fig. 3B). Similarly, a reactive cysteine residue in porcine prolyl oligopeptidase (POP) is not conserved in two prokaryotic POPs, represented in Fig. 3B by POP from *Aeromonas hydrophila* and *Flavobacterium meningosepticum*. This supports our contention that C256 is responsible for the inhibitory properties of IAA and NEM.

An excellent computer model of *E. coli* OpdB has been generated [16], based on the structure of porcine POP [17]. Employing a similar model of the *T. brucei* enzyme (Fig. 3A), we observed that C256 was brought into close proximity to residues E607 and E609. These two residues are key residues that bind to the P_1 substrate residue [10]. Thus, any chemical adduct that reacts and binds to C256 could interfere with the binding of the P_1-substrate residue to the E607/E609 pair. Alklyation of C256 by *N*-ethylmaleimide would introduce a large, bulky adduct [18], which could block substrate binding by steric hindrance. The situation is probably different for IAN (ICH_2COONH_2) and for IAA (ICH_2COO^-), since both molecules are very small, and have a similar size, but are very different with respect to OpdB inhibition properties. IAA was strongly inhibitory, while IAN was not. Alkylation of C256 by IAA would introduce a negatively charged CH_2COO^- adduct [19]. This would place an additional negatively charged cluster close to the E607/E609 pair, and may interfere with the correct binding or "docking" of the substrate at the active-site, perhaps placing the scissile bond in an unfavorable position for catalysis. In contrast, IAN would introduce a small neutral (i.e., uncharged) amide group at the same site, which does not appear to impede OpdB activity. In contrast to NEM, IAA and IAN, the pCMB was a much faster inhibitor of OpdB, and its activity was not restricted to C256. These data are consistent with the much more reactive nature of pCMB, in comparison with NEM, IAA and IAN [20]. Furthermore, pCMB alkylates both cysteines and free amines, perhaps explaining its more dramatic effect [20].

Since all of our cysteine to serine OpdB variants displayed catalytic properties similar to those exhibited by the WT enzyme (as reflected by the k_{cat} and K_m values for the hydrolysis of Cbz-Arg-Arg-AMC by each variant in the absence of reducing agents), it was unlikely that the cysteine to serine conversions caused structural changes in the enzyme that impaired catalytical capacity. However, since the replacement of some cysteine residues with serine residues in prolyl endopeptidase [21] and in thimet oligopeptidase [22] led to interference with

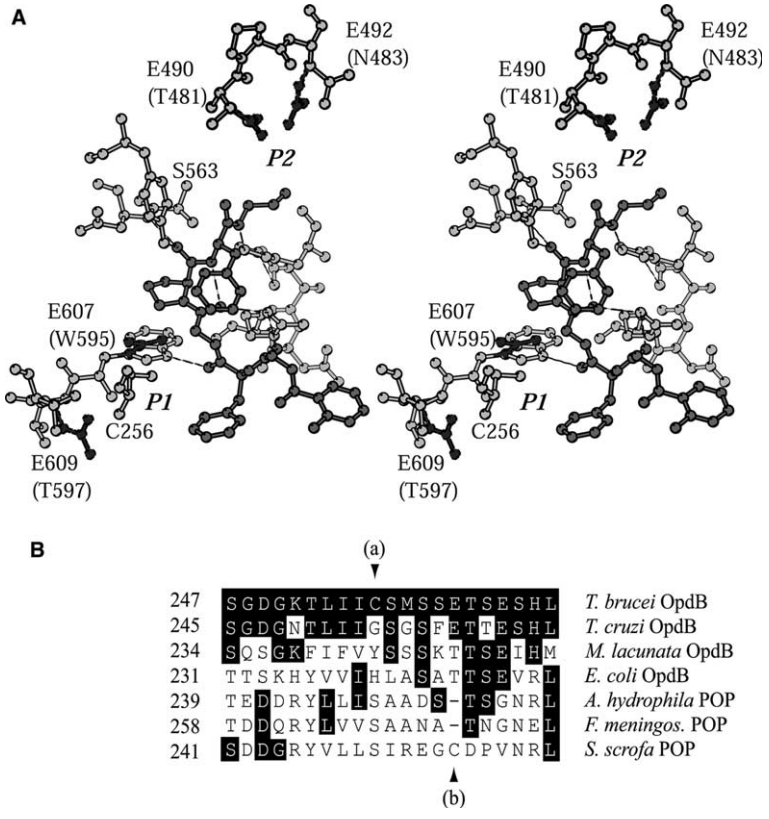

Fig. 3. Cysteine 256 in *T. brucei* OpdB. (A) Homology modeling of *T. brucei* OpdB, based on the closely related POP structure. This stereo model was prepared with MolScript exactly as described previously [12], using the coordinates of Protein Data Bank entry 1e8n. The substrate-binding carboxyl dyads of *T. brucei* OpdB: E607 and E609 (for P_1) and E490 and E492 (for P_2) are shown (in black) superimposed on the corresponding POP residues (POP residues are indicated in brackets), bound in a complex with an octapeptide substrate (*o*-aminobenzoyl-Gly-Phe-Gly-Pro-Phe-Gly-Phe(NO_2)-Ala-NH_2) that is shown darker than the oligopeptidase residues. The OpdB reactive cysteine residue C256 and the catalytic serine residue (S563), are also indicated. (B) Alignment of the region surrounding C256 for representative four members of the OpdB and POP subfamilies of serine peptidases. The full-length *T. brucei* OpdB [1] was aligned with the full-length OpdB sequences from *T. cruzi* [13], *M. lacunata* [15], *E. coli* [14], and the full-length POP sequences from *A. hydrophila* [26], *F. meningosepticum* [27] and *Sus scrofa* [28] using the CLUSTAL X alignment software [11]. The reactive *T. brucei* C256 is indicated by an (a), while a comparable reactive cysteine residue in POP [21] is indicated by a (b).

catalytic competence, we addressed whether this may be the case in our OpdB variants. Thus, we also constructed a C256A and a C256G variant, in addition to the C256S variant. However, in the case of OpdB, substitution of the reactive cysteine with alanine or glycine yielded similar kinetic data to those obtained with the C256S variant. We therefore concluded with confidence that C256 is the reactive cysteine residue responsible for the inactivation of OpdB by NEM and IAA. We have now initiated studies that will explore the possibility of using peptide-based OpdB substrates modified with thiol-reactive groups, to direct the alkylation of C256, thereby combining OpdB substrate specificity and thiol-alkylation properties to develop specific OpdB inhibitors.

A second property of OpdB that sets it aside from other serine peptidases is the ability of reducing agents to enhance catalytic activity. This is typically a property of cysteine peptidases, although some metallopeptidases, for example, thimet oligopeptidase [23], also exhibit activation by reducing agents. Thimet oligopeptidase forms covalently associated multimers through intermolecular disulfide bridges, with concomitant loss of catalytic power. Catalytic competence is regained upon reduction of multimers back to monomers with DTT [23]. Oligopeptidase B did not form dimers, or any other kind of multimers, in the absence or presence of reducing agents, which evident from its electrophoretic migration and chromatographic behavior under non-reducing and reducing conditions. Thus, the multimer ↔ monomer model does not apply to OpdB. It is not immediately apparent how the cysteine residues of OpdB mediate the enhancing effects of DTT and GSH. Reduction or rearrangement of intramolecular (i.e., within the same OpdB molecule) disulfide bridges may promote conformational changes with activity-enhancing effects, alternatively, the thiol groups of free cysteines may form mixed disulfides with small molecules, which are reduced to free thiols in the presence of DTT or GSH. The determination of the three-dimensional structure of OpdB, which is currently underway in our laboratories, may help to explain this activity-enhancing effect of reducing agents.

Could the thiol-sensitivity of OpdB be physiologically relevant? African trypanosomes contain millimolar concentrations of reducing agents in the cytosol. In *T. brucei*, assuming a cell volume of 5.8 μl per 10^8 cells [24], total cytosolic GSH concentrations exceed 1 mM, and this GSH exists either freely, or as a unique spermidine conjugate, trypanothione [N^1,N^8-bis(glutathionyl)spermidine] [25]. Thus, the trypanosome cytosol is a potently reducing environment, containing sufficient GSH to make the thiol-enhancing properties of OpdB physiologically relevant, a phenomenon that may exist to regulate OpdB activity in the parasites.

Acknowledgments: The authors express their thanks to Professor Luiz Juliano and Dr. Vitor Oliviera for expert advice and critical reading of the manuscript and to Drs. István Vadász and Oliver Eickelberg for outstanding support. This work was supported by grants from the Alexander von Humboldt Foundation (to R.M.), the Deutsche Forschungsgemeinschaft (to R.M.), the Human Frontiers Science Foundation (to R.M., V.F. and N.W.A.) and the National Institutes of Health (to N.W.A.). V.F. is a Royal Society University Research Fellow.

References

[1] Morty, R.E., Lonsdale-Eccles, J.D., Morehead, J., Caler, E.V., Mentele, R., Auerswald, E.A., Coetzer, T.H., Andrews, N.W. and Burleigh, B.A. (1999) Oligopeptidase B from *Trypanosoma brucei*, a new member of an emerging subgroup of serine oligopeptidases. J. Biol. Chem. 274, 26149–26156.

[2] Morty, R.E., Lonsdale-Eccles, J.D., Mentele, R., Auerswald, E.A. and Coetzer, T.H. (2001) Trypanosome-derived oligopeptidase B is released into the plasma of infected rodents, where it persists and retains full catalytic activity. Infect. Immun. 69, 2757–2761.

[3] Troeberg, L., Pike, R.N., Morty, R.E., Berry, R.K., Coetzer, T.H. and Lonsdale-Eccles, J.D. (1996) Proteases from *Trypanosoma brucei brucei*. Purification, characterisation and interactions with host regulatory molecules. Eur. J. Biochem. 238, 728–736.

[4] Morty, R.E., Pellé, R., Vadász, I., Uzcanga, G.L., Seeger, W. and Bubis, J. (2005) Oligopeptidase B from *Trypanosoma evansi*: a parasite peptidase that inactivates atrial natriuretic factor in the bloodstream of infected hosts. J. Biol. Chem. 280, 10925–10937.

[5] Morty, R.E., Troeberg, L., Pike, R.N., Jones, R., Nickel, P., Lonsdale-Eccles, J.D. and Coetzer, T.H. (1998) A trypanosome oligopeptidase as a target for the trypanocidal agents pentamidine, diminazene and suramin. FEBS Lett. 433, 251–256.

[6] Morty, R.E., Troeberg, L., Powers, J.C., Ono, S., Lonsdale-Eccles, J.D. and Coetzer, T.H. (2000) Characterisation of the antitrypanosomal activity of peptidyl alpha-aminoalkyl phosphonate diphenyl esters. Biochem. Pharmacol. 60, 1497–1504.

[7] Hemerly, J.P., Oliveira, V., Del Nery, E., Morty, R.E., Andrews, N.W., Juliano, M.A. and Juliano, L. (2003) Subsite specificity (S3, S2, S1′, S2′ and S3′) of oligopeptidase B from *Trypanosoma cruzi* and *Trypanosoma brucei* using fluorescent quenched peptides: comparative study and identification of specific carboxypeptidase activity. Biochem. J. 373, 933–939.

[8] Oleksyszyn, J., Boduszek, B., Kam, C.M. and Powers, J.C. (1994) Novel amidine-containing peptidyl phosphonates as irreversible inhibitors for blood coagulation and related serine proteases. J. Med. Chem. 37, 226–231.

[9] Morty, R.E., Authié, E., Troeberg, L., Lonsdale-Eccles, J.D. and Coetzer, T.H. (1999) Purification and characterisation of a trypsin-like serine oligopeptidase from *Trypanosoma congolense*. Mol. Biochem. Parasitol. 102, 145–155.

[10] Morty, R.E., Fülöp, V. and Andrews, N.W. (2002) Substrate recognition properties of oligopeptidase B from *Salmonella enterica* serovar Typhimurium. J. Bacteriol. 184, 3329–3337.

[11] Aiyar, A. (2000) The use of CLUSTAL W and CLUSTAL X for multiple sequence alignment. Methods Mol. Biol. 132, 221–241.

[12] Fülöp, V., Szeltner, Z., Renner, V. and Polgár, L. (2001) Structures of prolyl oligopeptidase substrate/inhibitor complexes. Use of inhibitor binding for titration of the catalytic histidine residue. J. Biol. Chem. 276, 1262–1266.

[13] Burleigh, B.A., Caler, E.V., Webster, P. and Andrews, N.W. (1997) A cytosolic serine endopeptidase from *Trypanosoma cruzi* is required for the generation of Ca^{2+} signaling in mammalian cells. J. Cell Biol. 136, 609–620.

[14] Kanatani, A., Masuda, T., Shimoda, T., Misoka, F., Lin, X.S., Yoshimoto, T. and Tsuru, D. (1991) Protease II from *Escherichia coli*: sequencing and expression of the enzyme gene and characterization of the expressed enzyme. J. Biochem. 110, 315–320.

[15] Yoshimoto, T., Tabira, J., Kabashima, T., Inoue, S. and Ito, K. (1995) Protease II from *Moraxella lacunata*: cloning, sequencing, and expression of the enzyme gene, and crystallization of the expressed enzyme. J. Biochem. 117, 654–660.

[16] Gérczei, T., Keserü, G.M. and Náray-Szabó, G. (2000) Construction of a 3D model of oligopeptidase B, a potential processing enzyme in prokaryotes. J. Mol. Graph. Model. 18 (7–17), 57–58.

[17] Fülöp, V., Böcskei, Z. and Polgár, L. (1998) Prolyl oligopeptidase: an unusual beta-propeller domain regulates proteolysis. Cell 94, 161–170.

[18] Riordan, J.F. and Vallee, B.L. (1972) Reactions with *N*-ethylmaleimide and *p*-mercuribenzoate. Meth. Enzymol. 25, 449–456.

[19] Benesch, R. and Benesch, R.E. (1962) Determination of –SH groups in proteins. Methods Biochem. Anal. 10, 43–70.

[20] Benesch, R. and Benesch, R.E. (1952) Reactions of thiols with organic mercury compounds. Arch. Biochem. Biophys. 38, 425–441.

[21] Szeltner, Z., Renner, V. and Polgár, L. (2000) The noncatalytic beta-propeller domain of prolyl oligopeptidase enhances the catalytic capability of the peptidase domain. J. Biol. Chem. 275, 15000–15005.

[22] Chen, J.M., Stevens, R.A., Wray, P.W., Rawlings, N.D. and Barrett, A.J. (1998) Thimet oligopeptidase: site-directed mutagenesis disproves previous assumptions about the nature of the catalytic site. FEBS Lett 435, 16–20.
[23] Shrimpton, C.N., Glucksman, M.J., Lew, R.A., Tullai, J.W., Margulies, E.H., Roberts, J.L. and Smith, A.I. (1997) Thiol activation of endopeptidase EC 3.4.24.15. A novel mechanism for the regulation of catalytic activity. J. Biol. Chem. 272, 17395–17399.
[24] Opperdoes, F.R., Baudhuin, P., Coppens, I., De Roe, C., Edwards, S.W., Weijers, P.J. and Misset, O. (1984) Purification, morphometric analysis, and characterization of the glycosomes (microbodies) of the protozoan hemoflagellate *Trypanosoma brucei*. J. Cell Biol. 98, 1178–1184.
[25] Fairlamb, A.H., Henderson, G.B., Bacchi, C.J. and Cerami, A. (1987) In vivo effects of difluoromethylornithine on trypanothione and polyamine levels in bloodstream forms of *Trypanosoma brucei*. Mol. Biochem. Parasitol. 24, 185–191.
[26] Kanatani, A., Yoshimoto, T., Kitazono, A., Kokubo, T. and Tsuru, D. (1993) Prolyl endopeptidase from *Aeromonas hydrophila*: cloning, sequencing, and expression of the enzyme gene, and characterization of the expressed enzyme. J. Biochem. 113, 790–796.
[27] Yoshimoto, T., Kanatani, A., Shimoda, T., Inaoka, T., Kokubo, T. and Tsuru, D. (1991) Prolyl endopeptidase from *Flavobacterium meningosepticum*: cloning and sequencing of the enzyme gene. J. Biochem. 110, 873–878.
[28] Rennex, D., Hemmings, B.A., Hofsteenge, J. and Stone, S.R. (1991) cDNA cloning of porcine brain prolyl endopeptidase and identification of the active-site seryl residue. Biochemistry 30, 2195–2203.